A3.3 Bacteriology Position Statements

A. 2000 – 2002 Position Statements

1. **Initial Position Statement**

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. The four bacterial fish pathogens considered in this chapter represent etiological agents which are known to exist in carrier states, but which have the potential for generating severe epizootics of clinical disease under the appropriate conditions. The methods are described for detection and identification of each pathogen in the absence of clinical signs. While other bacterial pathogens exist which can cause serious disease in fish, they are often widely distributed and cannot be controlled through transfer restrictions due to their ubiquitous nature (such as the pathogenic *Flavobacterium* species), and therefore, are not the focus of these inspection procedures.

The accurate identification of a bacterial species is based upon patterns of characteristics observed when live, pure bacterial isolates are cultured under a variety of environmental and biochemical conditions. All four bacterial fish pathogens considered for identification during a fish health inspection are culturable. All have been exhaustively characterized in a variety of widely recognized bacteriological manuals (Bergey's 1984; MacFaddin's 1980 and 2000; Austin and Austin 1987). The extensive characterization of these species has lead to the establishment of simple testing schemes for presumptive identification of bacteria isolated from fish tissues as described in these protocols. *Renibacterium salmoninarum* , however, is relatively fastidious and difficult to culture and characterize phenotypically in the period of time desired to accomplish the completion of a fish health inspection. Serological techniques are also considered to be rapid, highly specific means for achieving presumptive identification of bacteria. Because of its fastidious nature, the fluorescent antibody technique has been long developed as a presumptive screening tool for the detection of *R. salmoninarum* in fish tissues.

It is generally agreed that identification of a bacterial isolate based on phenotypic or serological characteristics alone poses the possibility that a population of fish be inaccurately labeled as diseased on a fish health inspection report. Although either method of identification is acceptable as a screen for pathogens in fish, neither technique alone is precise enough to distinguish between some similar organisms. For these reasons, it is always necessary to apply a second testing regime, referred to here as “confirmatory,” to establish the accuracy of the screening test. The protocols described in this document are presented in such a manner. In past decades, studies with nucleic acids and genetic methods have furthered the accuracy in the classification and identification of bacterial species. These tools, however, were limited to research because of the difficulty in applying them accurately under clinical situations. The more recent developments in polymerase chain technology, however, have revolutionized the use of molecular biology in pathogen detection in clinical laboratories. PCR is a practical, sensitive, and accurate means to confirm the presumptive identification of a bacterial pathogen by the isolation and amplification of segments of DNA existing within fish tissues. It is presented in these protocols as an alternative to time consuming selective culture for confirmation of positive *R. salmoninarum* FAT results.

B. 2002 – 2003 Position Statements

1. **Should *Piscirickettsia salmonis* be added to the handbook?**

   a. The bacteriology subcommittee agreed that *Piscirickettsia salmonis* inspection procedures should be included in the bacteriology chapter at this time because the pathogen has the potential for causing severe epizootics and it is of regulatory concern.

   b. Two screening methods were selected: (1) isolation in tissue cell culture without antibiotics and/or (2) identification of characteristic cells in stained tissue impressions. Inoculation of susceptible cells is the most sensitive method for detecting *P. salmonis*. However, because *P. salmonis* is sensitive to low levels of antibiotics typically used in cell culture, all cultures must be free of antibiotics. Samples collected aseptically in the field may easily become contaminated by other bacteria. For this reason, an additional screening method (Giemsa stained tissue impressions) was included.

   c. For confirmation of *P. salmonis,* serological (immunofluoresence or immunohistochemistry) and PCR procedures were included. Screening and confirmatory methods are consistent with those in the OIE Diagnostic Manual for Aquatic Animal Diseases.

   d. It was also recognized that the screening methods may not detect covert infections; however, these methods represent the best available at this time. A statement addressing limitations of the screening assays was included.

   e. The incubation times selected for tissue culture were selected based on published peer reviewed journal articles and the OIE Manual. The committee expressed concern the combined 42-day incubation was to long, but at this time there is no available information to make a change in the incubation time.

C. 2003 – 2004 Position Statements

1. **Should culture and confirmation procedures for *Flavobacterium psychrophilum*, the causative agent of coldwater disease be added to the inspection manual?**

   a. The bacteriology subcommittee agreed that culture and confirmation procedures for *Flavobacterium psychrophilum* should not be added to the inspection manual at this time. At present, there are no validated methods to screen fish for *F. psychrophilum*. An IFAT method is being developed that could be used on ovarian fluids and kidney smears for broodstock screening; a PCR method is also being developed; selective media are being developed to reduce contamination by other bacteria and fungi; and methods are being developed to detect the bacterium in water. Also, the distribution of *F. psychrophilum* is global, and it is naturally present in water and sediments. At present, no agency regulates this organism, however, antibiotic resistant strains of the bacterium are considered reportable in the Pacific Northwest.

   Antibiotic sensitivity protocols have not been standardized for F.p. When these tests, including sensitivity to antibiotics, have been validated, this question should be reconsidered.

2. **Should methods for Antimicrobial Sensitivity testing of bacteria be added to the inspection manual?**

    a. The bacteriology subcommittee agreed to add a chapter on antimicrobial sensitivity testing to the inspection manual.

    b. It is important to collect data on antibiotic sensitivity for fish pathogens because there is potential for agencies to regulate the movement of fish infected with resistant strains of bacteria. Since the disc diffusion protocols have already been peer reviewed and published, it would be very easy to incorporate them as a separate chapter in the inspection manual.

    c. The subcommittee felt this was the best place for the protocols (rather than the diagnostic section of the Blue Book) due to the potential for agencies to regulate pathogens based on their resistance to antibiotics (a standardized method would be needed for this).

3. **Should the requirement of growing bacteria in TSB prior to preparing the hanging drop motility test be omitted?**

    a. The bacteriology subcommittee agreed that bacteria do not need to be cultured in TSB before preparing a hanging drop suspension to test for motility, unless the bacteria appear non-motile.

    b. Colonies may either be grown in TSB (or other suitable broth) or taken from an agar surface and suspended in sterile saline to evaluate motility.

    c. If bacterial suspensions prepared from solid agar, or a stab of a semi solid agar appear non-motile, the bacteria should be cultured in TSB and the hanging drop method used to confirm motility.

4. **Should the procedures to distinguish *A. salmonicida salmonicida* from *A. salmonicida achromogenes* be revised?**

    a. The bacteriology subcommittee agreed to remove arabinose from the list of differential tests pertaining to *A. salmonicida salmonicida* and *A. salmonicida achromogenes*. Arabinose reactions were consistent among all references, however the test itself requires longer incubation and is difficult to read/interpret for A. sal. when commercially available agar is used.

    b. Indole, esculin and maltose tests were already added to the flow chart in the 2003 revision of the inspection manual, and the +/- for these tests are consistent with Bergey's manual. However, in the subcommittee's discussion, we discovered there are published references (Wiklund and Dalsgaard 1998; Wiklund and Dalsgaard 1995; Chapman et al. 1991; Austin and Austin 1987) indicating typical and atypical A. sal. do not always utilize indole and maltose according to Bergey's scheme. There is a need to compare the variability of *A. salmonicida salmonicida* and *A. salmonicida achromogenes* in utilizing indole, maltose, and esculin to determine whether these tests should be listed as secondary tests in the flowchart.

    c. If these tests are retained in the flowchart, companion text needs to be developed in the furunculosis chapter.

d. Other biochemical tests could be evaluated such as mannose, degradation of blood, or others to differentiate *A. salmonicida salmonicida* from *A. salmonicida achromogenes.*

e. The O/F box in the flow chart has K/AG, and A/AG reactions which seem to refer to TSI rather than O/F reactions. Perhaps using the words "fermenter" and "non-fermenter" or "oxidizer"should be used instead.

5. **Additional Comment**

The subcommittee recognized that specific research is needed to address several aspects of the diagnosis of fish bacterial pathogens. We list them here, so they are of record, but also suggest the inspection manual review team work with the technical standards committee with the goal of obtaining funding for labs to work on topics directly related to enhancing the inspection manual and Blue Book.

a. Develop screening techniques to detect *F. psychrophilum* in carrier fish. Some work has been started on IFAT and PCR methods, and there is a need to modify existing agars to enhance growth of F.p. and reduce fungal overgrowth on agar plates. There may also be selective or differential media that could be developed for F.p.

b. Although disc diffusion protocols have been developed for many fish pathogens, methods for *F. columnare* and *F. psychrophilum* have been problematic. Work continues at Louisiana State University for F. c., but to our knowledge, no one has worked to standardize a protocol for F.p. Once methods are developed, the NCCLS can coordinate field testing of the methods at fish health labs similar to what was done for other pathogens and disc diffusion protocols. If antibiotic resistant strains of F.p. are regulated in the future, there would be a need for a standardized antibiotic sensitivity method in the inspection manual.

c. Compare the variability of typical and atypical A. sal. isolates in their ability to utilize indole, maltose and esculin. In addition, evaluate other biochemical tests (such as those for mannose, lecithin or Tween 60 for example) or blood degradation to differentiate typical from atypical A. sal.

These needs arose from our discussion related to suggested changes for the inspection manual, but there may be research needs for other pathogens (protocol development, validations, etc.), which could be added to this list. The committees could brainstorm funding options and solicit specific researchers to do the work.

D. 2004 – 2005 Position Statements

No changes were suggested for Chapter 3, Bacteriology for the 2004-2005 revision year.

E. 2006 – 2007 Position Statements

1. **Should the procedures be amended to allow the use of pools of kidney and spleen tissues from up to 5 fish for detection of bacteria that are screened by plate culture?**

   The amended procedure would enable the use of samples collected for virological analyses to be used for certain bacteriological analyses as well, provided that no antibiotics were added to the samples. The request was based on a paper (S. Mumford, C. Patterson, J. Evered, R. Brunson, J. Levine and J. Winton. 2005. Comparison of individual and pooled sampling methods for detecting bacterial pathogens of fish. J. Vet. Diagn. Invest. 17:305-310) that described the equivalency of using pooled kidney-spleen samples (from 5 fish) to individual kidney sampling techniques for detection of *Yersinia ruckeri* in culture.

   Despite the potential advantages of sample pooling, the conclusion reached by the committee was that more data was needed from multiple species to validate the technique for all of the pathogens that would affected by the change. These pathogens include *Aeromonas salmonicida* and *Edwardsiella ictaluri* in addition to *Yersinia ruckeri*. No changes were made in this edition.

F. 2008-2010 Position Statements

1. **Should the procedures be amended to allow for the use of pools of kidney and spleen tissues from up to 5 fish for detection of bacteria that are screened by plate culture?**

   Upon further review, the committee has decided to allow the use of pooled kidney and spleen tissue from up to 5 fish for detection of bacteria that are screened by plate culture. The committee concluded that the publication above (S. Mumford, C. Patterson, J. Evered, R. Brunson, J. Levine and J. Winton. 2005. Comparison of individual and pooled sampling methods for detecting bacterial pathogens of fish. J. Vet. Diagn. Invest. 17:305- 310) could be used as a model for other culturable gram negative bacteria (*Aeromonas salmonicida* and *Edwardsiella ictaluri*). The committee weighed the possible disadvantages (contamination and possible loss due to dilutional effect) with the advantages, (using a homogenized tissue sample instead of a loop, the increased lab efficiency, and cost savings) and concluded that 5 pooling of kidney and spleen tissues as described above should be included in the procedures for bacterial culture.

G. 2011 – 2012 Position Statements

1. **Should the Y. ruckeri screening tests (3.3.A.1.f.iii) be changed to include non-motile isolates?**

   The committee agreed that non-motile variants of Y. ruckeri are well documented in the literature (Austin et al. 2003 System. Appl. Microbiol 26:127; Wheeler et al. 2009 Dis. Aquat. Org. 84:25; Welch et al. 2011 Appl. Environ. Micro. 77(10):3493). The screening methods were changed to address non-motile Y. ruckeri variants.

H. 2013 – 2014 Position Statements

No changes or reviews requested.

I. 2019-2020 Position Statements

Should the *Renibacterium salmoninarum* (*R. sal.*) qPCR assay (Chase et al. 2006) be adopted into Section 2 (Aquatic Animal Health Inspections) of the American Fisheries Society Fish Health Section Blue Book. This assay was designed to detect *R. sal.* DNA in fish tissues, ovarian fluid, or Bacterial isolates by the Western Fisheries Research Center.

a. A bacteriology sub-committee was assembled to evaluate the adoption of the real-time PCR assay described above. The subcommittee was chaired by Dr. Gavin Glenney and included Mohamed Faisal, Maureen Purcell, Ryan Katona, Ashley Malmlov, James Thompson, and Becca Wolking. The committee concluded that the adoption of this validated and widely tested molecular assay into the Fish Health Section Blue Book would be a needed first step to bring the existing document up to date and relevant in the world of aquatic animal molecular diagnostics. The advantages of real-time PCR over conventional PCR are numerous; including: quantification in exponential growth phase, reduced contamination (closed-tube, no post-PCR processing), extra level of specificity with probe technology, and increased dynamic range. Standard Operating Procedures (SOPs) for the real-time PCR assay has been written in formal Washington Animal Disease Diagnostic Laboratory (WADDL) SOP format to initiate a consistent new methodology for new molecular assays assimilated into the Blue Book.

b. The *R.sal.* sub-committee concluded that the current presumptive fluorescent antibody test (FAT) is sufficient as a presumptive assay in Section 2 (Aquatic Animal Health Inspections) and possessed the advantage of being able to visualize fluorescing bacterial cells of the correct shape and size. With this in mind, the sub-committee came to a majority that the *R.sal.* qPCR assay be adopted in a confirmatory role in Section 2 (Aquatic Animal Health Inspections) of the AFS Fish Health Section Blue Book. The assay would not replace the current nested PCR confirmatory assay but would serve as an alternative. This would allow laboratories that currently do not possess real-time PCR machines to continue using PCR for confirmation. Also, by providing the qPCR assay (Chase et al. 2006) as an option for *R.sal.* confirmation, fish health professionals can eliminate or bypass two negative characteristics of nPCR, a time consuming practice that offers an ever present risk of lab contamination.

c. In Chapter 3. Bacteriology of the Inspection Manual (3.5 Renibacterium salmoninarum), a caveat to the AFS one presumptive, one confirmatory assay recommendation is found as follows- (Exception: Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT). With this exception in mind, a minority of the *R. sal.* sub-committee proposed having the *R.sal.* qPCR assay (Chase et al. 2006) adopted as a presumptive/screening assay alternative in addition to FAT, as in a fit for purpose manner. This proposal was refuted and the majority of the subcommittee chose to have its role be strictly confirmatory.